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Uptake of Lead by a Ciliate, *Stylonychia mytilus*, Isolated from Industrial Effluents: Potential Use in Bioremediation of Wastewater

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Heavy metals in wastewater come from industries and municipal sewage, and they are one of the main causes of water and soil pollution. Concentration of lead in atmosphere is of serious environmental concern (Zelikoff et al. 1988). Lead contamination in surface water mainly comes from anthropogenic sources (96%), particularly from combustion of leaded fuels, pyrometallurgical non-ferrous metal production and coal combustion. Lead in natural waters may be in the form of organic lead complexes originally from the fuel of ever growing automobile population and subsequent break down of tetraethyl lead (Ashraf et al. 2002; Nriagu 1989).

Lead is a ubiquitous toxic metal which has mutagenic, carcinogenic, genotoxic, anthropogenic and phytotoxic effects (Alvarez et al. 2003; Zelikoff et al. 1988). Severe lead toxicity has been known to cause sterility, abortions and neonatal mortality and morbidity. The most serious effects of lead are related to central nervous system (Goyer 1993). It is considered as non-essential metal with no biological role in microorganisms, animals and plants (Bruins et al. 2000).

Because traditional cleanup processes of heavy metal contaminated soils and waters are expensive and practical only in small areas (Moffat 1995), researchers have looked for new cost effective technologies that include the use of microorganisms, biomass, and live plants (Cervantes et al. 2001; Rehman and Shakoori 2001; Ebbs and Kochian 1998; Haq and Shakoori 1998). Microbial metal removal has received much attention in the last years due to the potential use of microorganisms for cleaning metal-polluted water (Ledin 2000).

Protozoans have been found to be present in and metabolizing industrial effluents contaminated by toxic metal ions such as Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+} and Cd^{2+} and other toxic compounds (Haq et al. 1998; Madoni et al. 1996; Schlenk and Moore 1994).The long-term survival of protozoa in media containing relatively high concentrations of heavy metal ions shows that these organisms have strategies to tolerate, resist or detoxify organic substances and heavy metals (Shakoori et al. 2004; Haq et al. 2000).

One of the objectives of this study was to evaluate the survival of protozoa in

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media containing heavy metals such as Cd^{2+}, Pb^{2+}, Cu^{2+} and Cr^{6+} and determine the uptake of lead by these organisms. A number of authors have already emphasized the role of protozoa in wastewater treatment plants (Shakoori et al. 2004; Haq et al. 2000; Fernandez-Leborans et al. 1998; Madoni et al. 1996).

MATERIALS AND METHODS

Wastewater samples from a tannery effluent were collected in screw capped sterile bottles from Kasur (Pakistan). The pH and temperature of these samples were also recorded at the time of collection. The samples were inoculated in Bold-basal salt medium in 100 ml conical flasks (Haq et al. 1998). A large number of bacteria, yeast, algae, and various protozoa were present in the original wastewater sample.

For isolation of protozoa, antibiotics, *i.e.* ampicillin (25 µg/ml), chloramphenicol (35 µg/ml) and gentamicin (25 µg/ml), were used to prevent growth of bacteria. Algae were excluded by keeping the culture in semidarkness. Yeast was excluded by absence of any organic substance in the medium. Culture was plated to YEPD medium and no growth was appeared on the fungal medium. Axenic culture of protozoa was made according to Shakoori et al. (2004).

One hundred milliliters of different media, in 250 mL conical flasks, was inoculated under aseptic conditions with 10µL of inoculum containing 40-50 ciliates. The cultures were maintained in the laboratory for one week at room temperature (25-27°C). The growth of *Stylonychia* was observed in the cultures by counting the number of ciliates at regular intervals.

The growth curves of *Stylonychia* were determined in different media i.e. LB (2 % (w/v) proteose peptone and 0.1% Bacto yeast extract), Molasses medium (1% aqueous solution of molasses), Wheat and rice grain medium (1 boiled rice and wheat grain in 10mL of distilled water) and Bold-basal salt medium [$NaNO_3$ (0.25g/l), $CaCl_2.H_2O$ (0.025g/l), $MgSO_4.7H_2O$ (0.075g/), K_2HPO_4 (0.075g/l), KH_2PO_4 (0.175g/l), NaCl (0.025g/l), EDTA (0.05g/l), KOH (0.031g/l), $FeSO_4.7H_2O$ (0.04g/l), H_2SO_4 (0.001,L/l), H_3BO_3 (0.01142g/l), $ZnSO_4.7H_2O$ (0.00881g/l), $MnCl_2.4H_2O$ (0.00144g/l), MoO_3 (0.00071g/l), $CuSO_4.5H_2O$ (0.00157g/l) and $Co(NO_3).6H_2O$ (0.00049g/l)], diluted 1:1000 with distilled water, for 8 days. Glucose as carbon source was only added as 1g/L in Bold-basal salt medium. The pH of each medium was adjusted at 7.5. No metal ions were added in these media. The growth of culture was checked by counting the number of protozoan cells in the medium as described earlier (Haq et al. 1998).

Resistance of *Stylonychia* to four metal ions *i.e.* Cr^{6+}, Cu^{2+}, Pb^{2+} and Cd^{2+} was checked by addition of the respective metal salts ($K_2Cr_2O_7$, $CuSO_4.5H_2O$, $Pb(NO_3)_2$ and $CdCl_2$) in the Bold-basal salt medium. Metals ions were sterilized separately and added to the medium when the temperature of the salt medium was slightly less than 50°C. For Cr^{6+}, Cu^{2+}, and Cd^{2+} the concentration in the medium on the first day was 1µg/ml with an increase of 1µg/ml every day for 30 days for

Cr^{6+}, 20 days for Cu^{2+}, and 23 days for Cd^{2+}. For treatment with Pb^{2+} the concentration in the medium on the first day was 2 µg/ml of Pb^{2+} with an increase of 2 µg/ml of Pb^{2+} every day for 30 days. Although the death of protozoa is confirmed by the lysis of the cell, movement is considered to be a vital sign of life. When the protozoa became inactive, no more metal was added.

The effect of different metal ions on growth of the culture was checked by counting the number of protozoan cells in the medium. At least three counts were taken to get a mean of every reading. The growth was compared with that of the control culture, which contained no added metal ions. The activity, shape and size of the protozoans were also noted. The size was measured with an ocular micrometer after restricting the movement of the ciliates by putting the culture in methylcellulose and staining with 1% neutral red.

The metal processing capability of *Stylonychia* was checked by adding Pb^{2+} at a concentration of 10.0 µg/ml of Pb^{2+} in the culture medium. The control culture medium also contained Pb^{2+} at a concentration of 10.0 µg/ml but was without the ciliates. The cultures were incubated for 6 days and from each medium (control and treated) 5 mL culture was taken out under sterilized conditions after 0, 48, 72, 96 hours, respectively. The cultures were spun down at 3000 rpm for 15 min and the supernatants were used for the estimation of Pb^{2+} by atomic absorption spectrophotometer (Varian, U.S.A) at wavelength 217.0 nm. The amount of Pb^{2+} in the supernatants was determined using standard curve. The percentage reduction in the amount of Pb^{2+} in the medium was calculated.

RESULTS AND DISCUSSION

The growth curve pattern of *Stylonychia* was obtained by counting the number of cells in the culture every day for 8 days. There was a gradual increase in the number of cells in each culturing medium. The number of cells increased from 66 to 620 cells/ml in LB medium, from 83 to 300 cells/ml in 1% Molasses medium, from 87 to 983 cells/ml in Wheat and Rice medium and from 84 to 1133 cells/ml in Bold-basal salt medium. A large number of media have been tried and reported for the growth of protozoa. Generally it is tedious to grow protozoa in the laboratory due to special organic supplements needed in the medium for their growth (Weekers and Vogels 1994). This laboratory has already reported the growth of two protozoan species in a medium containing salts only (Haq et al. 1998). In this study *Stylonychia* has been successfully grown in the Bold-basal salt medium. This finding would elucidate new trends in culturing protozoa. The growth curves are shown in Fig.1.

Stylonychia mytilus was found to be resistant to Pb^{2+} at a concentration of 60 µg/mL. The Pb-resistant ciliate was also found to tolerate Cu^{2+}, Cr^{6+} and Cd^{2+} at concentrations of 20 µg/mL, 30 µg/mL and 23 µg/mL, respectively. There was apparently no reduction in the size of *S. mytilus* cells. Movement, which is a vital sign of life, was taken as a parameter of metal toxicity. The movements of the ciliate slowed down in the presence of $K_2Cr_2O_7$ (30 µg/mL) but almost stopped in

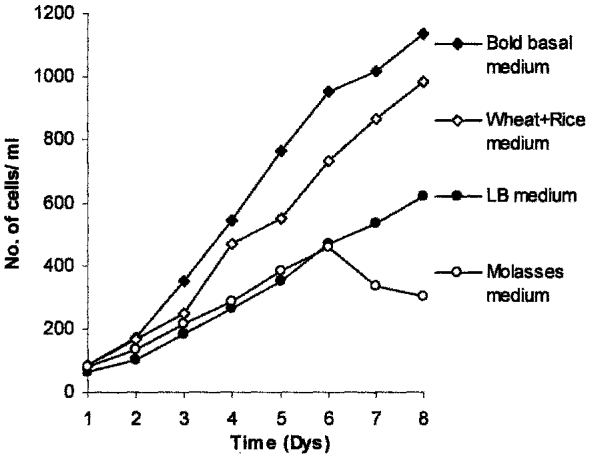

Figure 1. Growth curves of *Stylonychia mytilus* in different media containing no metal ions.

the presence of $CuSO_4$ (20 μg/mL) and $CdCl_2$ (23 μg/mL). The presence of Pb $(NO_3)_2$ (60 μg/mL) did not have any significant effect on the movement of ciliates. The order of resistance on the basis of motility was $Pb^{2+}>Cr^{6+}>Cd^{2+}>Cu^{2+}$.

Mitotic activity, which is indicated by cell population, was adversely affected by the presence of metal ions in culture media. The control culture contained $1.42x10^3$ cells/ml on day 1, which decreased to$1.25x10^3$ cells/ml after 30 days. However, when Cu^{2+} (20μg/ml) was added the number decreased from $1.33x10^3 \pm 3.30$ to $0.85x10^3 \pm 2.83$ cells/ml in 20 days ($P < 0.001$). In the presence of Pb^{2+} (60 μg/ml) the number of cells decreased from $1.38x10^3 \pm 3.30$ to $1.02x10^3 \pm 2.36$ cells/ml ($P < 0.001$), $1.30x10^3 \pm 3.30$ to $0.90x10^3 \pm 3.40$ cells/ml in Cr^{6+} (30 μg/ml) ($P < 0.001$) after 30 days, whereas the number of cells decreased from $0.8x10^3 \pm 2.83$ to $0.36 x10^3 \pm 1.89$ cells/ml in the presence of Cd^{2+} (23 μg /ml) ($P<0.001$) in 23 days. The reduction in the cell population was 36% (Cu^{2+}), 26% (Pb^{2+}), 31% (Cr^{6+}) and 55% (Cd^{2+}), respectively. The order of resistance regarding the reduction in the number of the cells was, therefore, $Pb^{2+}>Cr^{6+}>Cu^{2+}>Cd^{2+}>$.

Stylonychia could efficiently process Pb^{2+} from the medium. The protozoan culture grown in medium containing lead (10.0μg/ml) could reduce 80% of lead from the medium after 48 hours, 82% after 72hours and 86% after 96 hours, respectively (Fig.2).

It is well recognized that microorganisms have a high affinity for metals and can accumulate both heavy and toxic metals by a variety of mechanisms (Pas et al. 2004; Yilmaz 2003; Shakoori and Muneer 2002; Ledin, 2000; Shuttleworth and Unz 1993). These have been used to remove metals from polluted industrial and domestic effluents on a large scale. Microorganisms have a high surface area-to-

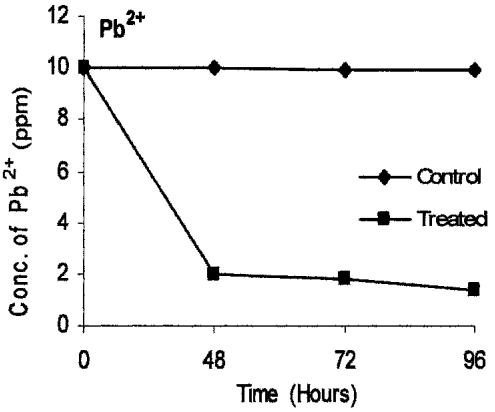

Figure 2. Uptake of Pb^{2+} by *Stylonychia mytilus* growing in Pb^{2+} containing medium. The control did not contain cells of the isolate.

volume ratio because of their small size and therefore provide a large contact area that can interact with metals in the surrounding environment (Ledin 2000). Microbiological detoxification of polluted water is economical, safe, and sustainable (Eccles 1995).

Metal resistant protozoa have been reported in wastewaters and metal-polluted environments (Shakoori et al. 2004; Haq et al. 2000; Madoni et al. 1996). Accumulation of heavy metals in protozoa, as in higher animals, induces metallothionein synthesis (Piccinni et al. 1987). Metallothioneins are low molecular weight, metal-binding and cysteine rich proteins. The functions of metallothioneins have been proposed to involve roles of metal metabolism such as transport, storage and especially detoxification of heavy metals (Tohoyama et al. 1995).

Shakoori et al. (2004) reported that *Vorticella microstoma* showed remarkable ability to pick up heavy metal ions from the culture medium. The concentration of Zn^{2+} and Cr^{6+} was reduced 99% and 48% after 192 hours, respectively. These microorganisms actively contribute to the amelioration of the effluent quality, since the majority of them feed upon dispersed bacteria (Madoni 2000).

Ciliates, essential components of nearly all ecosystems, are attractive models for toxicological and ecotoxicological studies, due to their relative ease of culturing, short life cycle, cosmopolitan distribution, and sensitivity to environmental changes. In this study we have reported the isolation of *Stylonychia mytilus* which is resistant to highly toxic metal ions. This capability of the organism can be exploited for metal detoxification operations.

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